IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of:	Slepnev	Examiner:	Horlick, K.
Serial No.:	10/719,185		
Filed:	November 21, 2003	Group Art Unit:	1637
Entitled:	Sampling Method and Apparatus for Amplification Reaction Analysis		
		Conf. No.:	3232

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

DECLARATION OF DR. VLADIMIR SLEPNEV UNDER 37 C.F.R. 1.132

I declare:

1. I, Vladimir I. Slepnev, hold the position of Chief Scientific Officer at Primera BioSystems, Inc., Mansfield, MA.

2. I hold a Master's Degree in Chemistry from Moscow State University and a Ph.D. in Biochemistry from the Russian Research Center of Molecular Diagnostics and Therapy (Moscow, Russia). I have performed post-doctoral research at Institute Pasteur (France) and at Yale University (New Haven, CT), where I also worked as a junior faculty member. I am an author on 32 peer-reviewed literature publications. A copy of my Curriculum Vitae is attached.

3. I am an inventor on the above-noted U.S. patent application.

4. I have read the Final Office Action issued April 30, 2007 in the above-noted patent application, and I understand that the Examiner has rejected claims 1, 4-35 and 38-63 as being obvious over Wiesner (Nucleic Acids Research 20: 5863-5864) in view of Schumm et al (U.S. Patent No. 6,479,235), and claims 35 and 38-61 as being obvious over Wiesner et al. in view of Brenner (U.S. Patent No. 6,228,589) and Schumm et al. Specifically, the Examiner stated:

> These claims are drawn to methods of quantitatively amplifying a plurality of nucleic acids, wherein an aliquot of the amplification mixture is dispensed or withdrawn at plural stages during the amplification regimen, further wherein at

least five different amplification templates are used, and in some embodiments wherein capillary electrophoresis is utilized for separation.

Wiesner teaches methods of quantitatively amplifying a plurality of nucleic acids, wherein an aliquot of the amplification mixture is dispensed or withdrawn at plural stages during the amplification regimen; see entire document on pages 5863-5864.

Wiesner does not disclose use of at least five different amplification templates, nor the use of capillary electrophoresis for nucleic acid separation.

Schumm et al. disclose the well-known technique of multiplex amplification wherein a plurality of different nucleic acid targets (at least thirteen; see abstract) are simultaneously amplified, and that electrophoresis, preferably capillary electrophoresis, is used to separate the different products produced by multiplex amplification.

One of ordinary skill in the art would have been motivated to use a plurality of amplification targets, such as at least five, as well as the technique of capillary electrophoresis, in the method of Wiesner, because the advantages of both multiplex amplification and capillary electrophoresis were well known and common knowledge in the art, as demonstrated by Schumm et al. In other words, the skilled artisan considering these references would have been motivated to apply multiplex amplification and capillary electrophoresis as taught by Schumm et al. in the method of Wiesner to provide the obvious advantage of facilitating quantitative amplification profiles of large numbers of target nucleic acids.

5. I have read the Wiesner, Schumm et al. and Brenner references. Based on the following analysis of the Wiesner reference, even as potentially modified by the teachings of Schumm et al., I cannot agree with the conclusion that the claimed invention is obvious over the Wiesner reference alone or in combination with Schumm et al. alone or with the teachings of Brenner.

A) First, the method described by Wiesner relies upon the removing of a known volume of reaction sample containing radioactive nucleotides at subsequent cycles of PCR, separating the entire aliquot by electrophoresis, measuring radioactivity incorporated in the PCR products, plotting the calculated copy number at each cycle and then extrapolating the resulting function to the cycle zero to obtain the initial number of nucleic acid target molecules in the sample. The described methods relies on two key conditions: knowledge of the exact volume of the aliquot subjected to separation and the ability to measure precisely the absolute quantity of the PCR product band at the given cycle. The absolute determination of target template taught by Wiesner will not work when applied to capillary electrophoresis and, particularly with

fluorescent detection, because these key conditions will not be satisfied as required for the linear regression calculations that are central to the Wiesner method.

Wiesner teaches:

> " Aliquots of 1 ul were taken from the reaction after consecutive cycles and run on 15% polyacrylamide gels, which were stained with ethidium bromide...The two product bands ... were isolated from the gel, slices were trimmed, dried at 80°C overnignt in liquid scintillation vials and the incorporated radioactivity was determined by liquid scintillation counting.... The concentration of the product accumulating in consecutive cycles, Nn (moles/ul) can be calculated from the incorporated radioactivity (cpm/ul), the specific radioactivity of the precursor dCTP (cpm/mol) in the reaction mixture and the number of dCTPs which can be incorporated into the newly synthesized stretch of the product *Y*, according to Nn (moles/ ul) = (cpm/ul) / ((cpm/mol)**Y*)). The initial concentration of double stranded DNA template at cycle zero, No (moles/ul) and the efficiency of amplification eff, can then be calculated by linear regression analysis of the transformed equation describing product accumulation in the PCR.
>
> Log Nn= log eff*n + No....."

It is clear from the description that the method is based on the exact knowledge of the volume of the aliquot subjected to separation, which becomes the basis for all calculations. Capillary electrophoresis (CE) differs from slab gel electrophoresis in the way that the sample is loaded into the separation medium. Whereas the entire volume of the aliquot placed into the slab gel goes into the separation, only a small and unknown portion of the aliquot placed for injection into the capillary actually enters the capillary.

In capillary electrophoresis there two ways to inject sample into a capillary: hydrodynamic injection and electrokinetic injection. Hydrodynamic injection, where sample is drawn into the capillary by creating a pressure differential between inlet and outlet ends of the capillary, is rarely used for nucleic acid separation due to the high viscosity of sieving polymer gels. The viscosity makes it very difficult to control the volume of the injected sample. It is nearly impossible to implement this mode of injection for multicapillary systems which are the preferred approach for nucleic acid CE.

In the electrokinetic mode of injection, a capillary is dipped into the sample aliquot together with an electrode, and by applying an electric field to the sample, negatively charged

nucleic acids enter the capillary and migrate toward positively charged electrode placed at the outlet of the capillary. The amount of the nucleic acids in the sample actually injected into the capillary depends on the duration of the injection, potential of the applied electric field, ionic composition of the sample (components of PCR mixture: salts, nucleotides and primers compete for injection with nucleic acids),"stacking" effect at the border of separating polymer and sample solution, and electroosmotic force moving the liquid enclosed in the capillary in the opposite direction in the electric field. The upper limit on the amount of injected material is imposed by the requirement to maintain reasonable resolution of the separation (the "length" of injected solution plug should be a small fraction of the capillary length). Technically, it is impossible to inject a sample with a volume equivalent of more than 100 nl. More importantly, the exact "volume" of the injected sample using electrokinetic injection is unknown. To adapt the method of Wiesner to the use of CE in place of slab gel electrophoresis, one would have to measure the volume of the sample injected during CE, however the Wiesner reference does not provide a teaching for how to do that, and neither do the Schumm or Brenner references. As discussed above, such a measurement may well not be possible.

B) Second, in addition to requiring knowledge of the exact volume of sample electrophoresed, the method of Wiesner requires precise measurement of the absolute amount of amplified PCR product contained within a separated peak. In the method described by Wiesner, radioactive label is used to measure incorporation in the PCR product of interest. It is conceivable to consider that a direct adaptation of Wiesner's method to capillary electrophoresis could include an approach which would measure the amount of radioactive label in the peaks separated by CE. This could be accomplished by using an in-line radioactive detector or by collecting fractions during CE and then measuring radioactive label in the collected fractions corresponding to the specific PCR product. However, to my knowledge, no in-line radioactive detectors exist yet for CE separation of nucleic acids, and so far fraction collection for CE is used for qualitative rather than quantitative assessments of nucleic acids.

Another option for absolute quantification of nucleic acid in the separated peaks is UV photometry. Unfortunately, the small optical path of the capillary (50-100 microns ID) prohibits the use of this detection method for applications requiring highly sensitive detection, such as monitoring of PCR amplification.

The preferred detection method in CE separation of nucleic acids is fluorescent detection. This method, by definition, does not provide absolute quantification, and therefore it uses additional standards of known abundance for relative quantitative measurement of target analytes. In order to adapt the quantitation method of Wiesner to use capillary electrophoresis in place of slab gel electrophoresis, one would need to provide a reference standard or multiple standards comprising the same type of fluorophore with identical excitation and emission properties to the target PCR amplicon. Since nucleotide sequence context is well known to affect properties of fluorescent labels, special reference standards would have to be developed for each individual amplicon present in the same multiplex reaction. Wiesner, or Schumm or Brenner, for that matter, do not teach design and validation of such standards. Moreover, I am not aware of any published reference that would teach practical implementation of such standards for absolute quantification of nucleic acids by capillary electrophoresis.

It is understood from the Final Office Action that the Wiesner reference "is cited not for the particulars of the multiplex amplification reaction, but for the teaching of quantitatively amplifying a plurality of nucleic acids, wherein an aliquot of the amplification mixture is dispensed or withdrawn at plural stages during the amplification regimen" (emphasis in the Office Action). It is my strong opinion that quantitation as taught by Wiesner cannot be achieved using capillary electrophoresis. It is my strong opinion that the method of Wiesner requires specific means described in the reference and that capillary electrophoresis cannot be simply substituted for slab gel electrophoresis if one expects to use the template nucleic acid quantitation approach that is central to the teachings of the Wiesner reference.

6. In view of the above, the proposed combination of teachings of Wiesner and Schumm et al. that are central to each of the claim rejections will not provide function necessary to meet the requirements of the claimed invention.

7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and

that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

07/24/2007

Date

Vladimir I. Slepnev